Journal of Chromatography, 420 (1987) 158–162
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3742

Note

Use of ion-exchange chromatography and high-performance liquid chromatography to prepare a substrate for differential assay of human pancreatic and salivary α-amylases in serum

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(First received February 3rd, 1987; revised manuscript received April 6th, 1987)

The differential assay of human pancreatic and salivary α-amylases (EC 3.2.1.1) in serum is important in clinical diagnosis because some diseases cause an increase in one of these amylases. For example, only the pancreatic α-amylase increases from pancreatic lesions such as acute pancreatitis. *p*-Nitrophenyl O-6-deoxy-6-[(2-pyridyl)amino]-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-α-D-glucopyranoside (FG5P) is a suitable substrate for a differential colorimetric kinetic assay of the two α-amylases with maltase (EC 3.2.1.20) and isomaltase (EC 3.2.1.10) as the coupled enzymes [1]. Its preparation includes: (1) chemical modification of amylose to F-amylose; (2) digestion of the modified amylose by glucoamylase and α-amylase to O-6-deoxy-6-[(2-pyridyl)amino]-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucose (FG6), other maltooligosaccharides with modified non-reducing-end glucose, and glucose; (3) isolation of FG6 from the digest by ion-exchange chromatography on a Dowex 50 column; (4) conversion of FG6 into FG5P by cyclodextrin glucanotransferase (CGTase) (EC 2.4.1.19) in the presence of *p*-nitrophenyl α-glucoside; (5) isolation of FG5P from reaction mixture by gel permeation on a Bio-Gel P-2 column and high-performance liquid chromatography (HPLC) on a C_{18} reversed-phase column [2,3]. We wanted to improve this procedure by omitting both the digestion with α-amylase and the chromatography with Dowex 50 and by using ion-exchange chromatography instead of the gel permeation with Bio-Gel P-2 for large-scale production. This paper reports the isolation and purification of FG5P by ion-exchange chromatography and HPLC.

0378-4347/87/$03.50

EXPERIMENTAL

Materials

A crude preparation of CGTase of *Bacillus macerans* was purchased from Amano Pharmaceutical (Nagoya, Japan). The activity of the enzyme is defined as previously reported [4]. Glucoamylase of *Rhizopus niveus* was purchased from Seikagaku Kogyo (Tokyo, Japan). Amylose A (mean molecular mass, 2000) was from Nakarai Chemicals (Kyoto, Japan).

Preparation of F-amylose

The chemical conversion of amylose into F-amylose was slightly modified from that reported previously [2]. The C-6 CH_2OH of the glucose residues of amylose was partly oxidized at a three-fold higher concentration of amylose than that reported previously, and the F-amylose produced was isolated from the reaction mixture by precipitation with ethanol instead of gel permeation on a Bio-Gel P-4 column. F-amylose was precipitated from the reaction mixture (100 ml, from 3 g of amylose) by the addition of 500 ml of ethanol. The F-amylose collected by centrifugation was dissolved in 100 ml of water and precipitated by the addition of 500 ml of ethanol. The precipitated F-amylose was dried under reduced pressure to remove ethanol and dissolved in water, bringing the volume up to 70 ml.

Enzymic conversion of F-amylose into FG5P

The F-amylose solution (70 ml) with the pH adjusted to 4.8 with 12 *M* hydrochloric acid was incubated with glucoamylase (20 mg) at 37°C for 20 h, and 3 ml of 1.3 *M* sodium borohydride in 0.5 *M* sodium hydroxide were added to the digest. The solution was left for 20 min at room temperature to reduce glucose liberated by glucoamylase to glucitol. The remaining sodium borohydride was decomposed by the addition of 12 *M* hydrochloric acid, and then the pH of the reaction mixture was adjusted to 5.5 with 4 *M* sodium hydroxide. *p*-Nitrophenyl α-glucoside (1.2 g) was dissolved in the solution, and the mixture was incubated with 4.3 U of CGTase at 37°C for 3 h. The enzymic reaction was stopped by the addition of 12 *M* hydrochloric acid to bring the pH of the solution to 3.0.

SP-Sephadex C-25 chromatography

The above enzymic reaction mixture was diluted with water to 1 l, and 330 ml of the solution were put on a column of SP-Sephadex C-25 (124×1.8 cm) equilibrated with 0.01 *M* pyridine–acetic acid buffer (pH 5.5). After being washed with 200 ml of the same buffer, the column was developed by a linear gradient generated by mixing 1.5 l of this buffer with 1.5 l of 0.3 *M* pyridine–acetic acid buffer (pH 5.5). The elution was monitored by measurement of the absorbance at 320 nm arising from 2-pyridylamino and *p*-nitrophenyl groups. The fractions of FG5P and *p*-nitrophenyl O-6-deoxy-6-[(2-pyridyl)amino]-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-α-D-glucopyranoside (FG4P) were identified by analytical HPLC as described below. The FG5P fraction was lyophilized and dissolved in 8 ml of water.

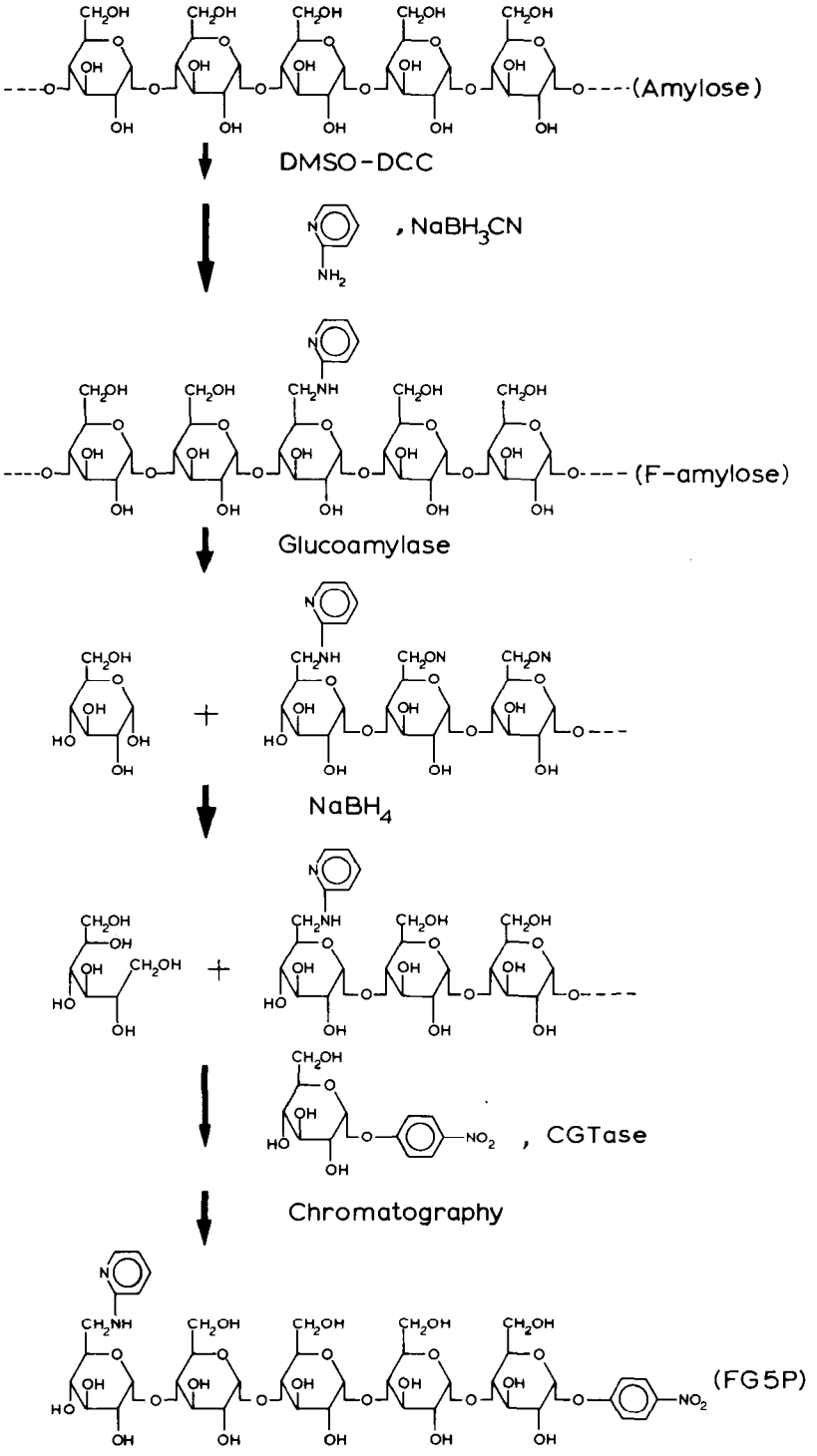

Fig. 1. Preparation of FG5P.

High-performance liquid chromatography

The HPLC system consisted of a Hitachi liquid chromatograph Model 655 pump, a Rheodyne injector Model 7125 with 900-μl sample loop, a Hitachi variable wavelength UV monitor Model 638-41 and an SIC chromatocorder 11. For analytical purposes, a Silicagel Z ODS-5C column (C_{18} reversed-phase, 150×4.6 mm, Wako, Osaka, Japan) was eluted at a flow-rate of 1.5 ml/min with 0.05 *M* ammonium acetate buffer (pH 5.0) containing 1.5% 1-butanol. For purification of FG5P, a D-ODS-5 YMC column (C_{18} reversed-phase, 25×2 cm, Yamamura, Kyoto, Japan) was used. The FG5P fraction (8 ml) isolated by SP-Sephadex C-25 chromatography was adsorbed on this column equilibrated with 0.05 *M* ammonium acetate buffer (pH 4.0) containing 0.05% 1-butanol. The column was washed with the same buffer for 40 min and developed with 0.05 *M* ammonium acetate buffer (pH 5.0) containing 1.5% 1-butanol at a flow-rate of 9.0 ml/min.

RESULTS AND DISCUSSION

The preparation of FG5P is summarized in Fig. 1. Sodium borohydride reduction was done to decompose the glucose liberated, because glucose competes with *p*-nitrophenyl α-glucoside as an acceptor on the next transglycosylation reaction by CGTase. The action of CGTase on the mixture of the limit-dextrins and *p*-nitrophenyl α-glucoside gave two transfer products, FG5P and FG4P. In addition, the enzymic reaction mixture contained various limit-dextrins with 6-deoxy-6-[(2-pyridyl)amino]-D-glucose residues, non-ionic oligo saccharides, glucitol and *p*-nitrophenyl α-glucoside. FG5P was separated by ion-exchange chromatography on an SP-Sephadex C-25 column as shown in Fig. 2.

Non-ionic compounds were not adsorbed on the column. Among the adsorbed

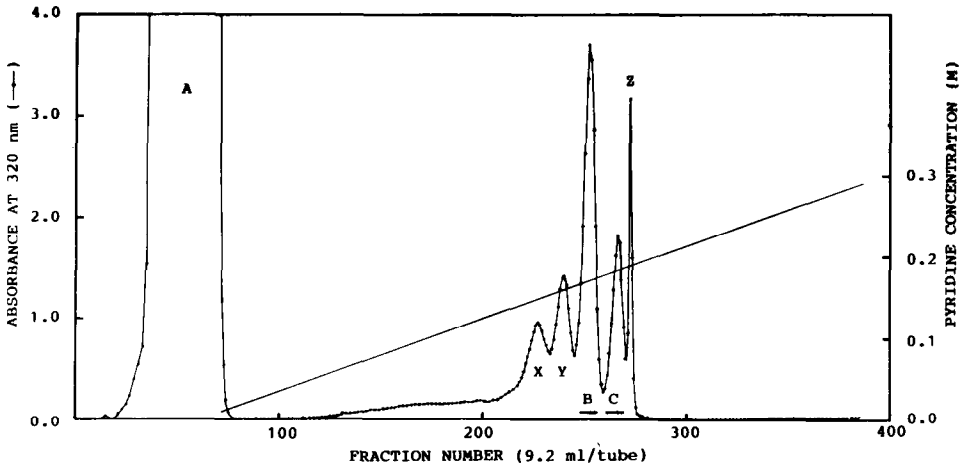

Fig. 2. Isolation of FG5P by SP-Sephadex C-25 chromatography. Details of the preparation of the sample loaded on the column and of the elution are described in Experimental. The absorbance was measured with a 0.5-cm path cell. Peaks: A = *p*-nitrophenyl α-glucoside (and glucitol and also non-ionic oligosaccharides); B = FG5P fraction; C = FG4P fraction; X, Y, and Z are mixtures of oligosaccharides with a 2-pyridylamino group(s).

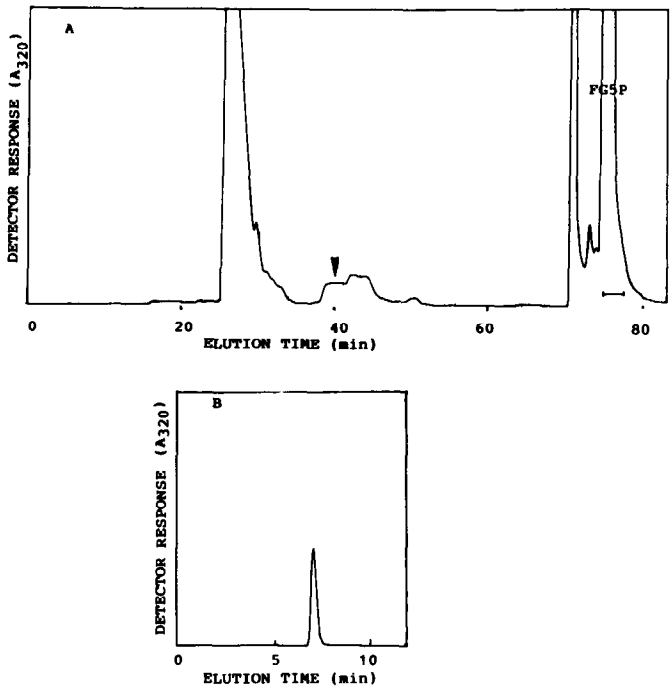

Fig. 3. Preparative HPLC of the FG5P fraction isolated by SP-Sephadex C-25 chromatography and analytical HPLC of FG5P obtained by preparative HPLC. Elution was performed as described in Experimental. (A) Preparative HPLC of the FG5P fraction isolated by SP-Sephadex C-25 chromatography. The fraction indicated by the bar was collected as FG5P. The arrow indicates the change of elution buffer. (B) Analytical HPLC of the FG5P fraction obtained by preparative HPLC.

compounds with a 6-deoxy-6-[(2-pyridyl)amino]-D-glucose residue, FG4P and FG5P were eluted rather late because of interaction between the *p*-nitrophenyl group and the Sephadex. The FG5P fraction was adsorbed on the preparative C_{18} reversed-phase column, and the column was eluted as described in Experimental (Fig. 3A). HPLC of the FG5P obtained showed that this adsorption chromatography purified the FG5P (Fig. 3B): 15 mg of pure FG5P were obtained from 1 g of amylose. The yield was four-fold that of the previous method. Furthermore, the preparation step was shortened, which makes possible the large-scale production of this substrate for use in the routine clinical assay of α-amylases. The objective of the study was achieved because ion-exchange chromatography and HPLC separated FG5P effectively.

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